Comparison of eosin-thiazin and Papanicolaou-Shorr staining for endometrial cytologies of broodmares. Technical note

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Abstract: INTRODUCTION: Exfoliative endometrial cytology is an easy and valuable diagnostic tool for the detection of inflammatory processes of the uterus that correlates well to culture results. The practical use of this procedure is limited due to the time-consuming Papanicolaou-Shorr staining technique. In this study the suitability of the rapid eosin-thiazin staining for endometrial smears was investigated. MATERIAL AND METHODS: Sample collection was carried out with a guarded culture swab (Knudsen-catheter) in 27 broodmares during routine gynaecological examination. Two smears were prepared from each collection. One was stained according to Papanicolaou-Shorr and the second using the eosin-thiazin method (Hemacolor®, Merck). Specimens with more than 0.5% neutrophil granulocytes were classified as positive for endometritis. The presentability of polymorphonuclear neutrophils (PMN), proportion of positive/negative samples, as well as the PMN contents were compared between the two staining methods. RESULTS: PMN were easily identifiable in both specimens. In Papanicolaou-Shorr stained smears 10 samples showed $>$ 0.5% neutrophil granulocytes (37%), whereas in the eosin-thiazin staining 12 samples were positive (44%). Thus results corresponded in 25 mares (95%). CONCLUSION: Eosin-thiazin staining is a suitable staining method for endometrial smears of broodmares, which surpassed Papanicolaou-Shorr method in two cases. CLINICAL RELEVANCE: The use of eosin-thiazin staining provides a considerable gain of time and renders endometrial cytology more attractive for routine stud farm practice.

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Technical Note: Comparison of Eosin-Thiazin and Papanicolaou-Shorr staining for endometrial cytologies of broodmares

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Key words

Endometritis, neutrophil granulocytes, PMN, endometrial swab, staining methods

Zusammenfassung

Gegenstand und Ziel: Die Untersuchung einer Endometriumszytologie ist ein wertvolles diagnostisches Hilfsmittel im Rahmen der Endometritisdiagnostik, welches jedoch in Deutschland bisher keinen festen Platz in der Routinediagnostik gefunden hat. Eine mögliche Ursache hierfür stellt das zeitaufwändige Färbeverfahren nach Papanicolaou-Shorr dar. Im Rahmen dieser Studie sollte der Einsatz der Eosin-Thiazin-Schnellfärbung für endometriale Abstriche gestestet werden, um diese Methodik in der Routineuntersuchung zu vereinfachen. **Material und Methoden:** Die exfoliativen endometrialen Zytologien wurden von 27 Stuten, die zur gynäkologischen Untersuchung vorstellig waren, mittels Knudsenkatheter entnommen. Die Tupfer wurden doppelt ausgestrichen und je einer nach Papanicolaou-Shorr und einer nach Eosin-Thiazin (Hemacolor®, Merck) gefärbt. Verglichen wurde die Detailerkennbarkeit und die Darstellung von neutrophilen Granulozyten. Waren mehr als 0,5 % neutrophile Granulozyten darstellbar, wurde die Zytologie als positiv gewertet. **Ergebnisse:** Neutrophile Granulozyten waren in beiden Färbungen eindeutig darstellbar. In der Papanicolaou-Shorr-Färbung waren zehn Stuten zytologisch positiv (37 %) und in der Eosin-Thiazin-Färbung zwölf Stuten (44 %). Eine Übereinstimmung des Ergebnisses zytologisch positiv oder negativ ergab sich beim Vergleich der Färbemethoden in 25 Fällen (95 %), wobei bei zwei Stuten ausschließlich in der Eosin-Thiazin-Färbung neutrophile Granulozyten nachweisbar waren. Ein geringgradig höherer Gehalt an neutrophilen Granulozyten in der Papanicolaou-Shorr-Färbung konnte in drei Fällen nachgewiesen werden. **Schlussfolgerung:** Die Ergebnisse dieser Studie zeigen, dass die Eosin-Thiazin-Färbung für exfoliative Endometriumszytologien geeignet ist, sogar in 2 Fällen der Papanicolaou-Shorr Färbung hinsichtlich des Nachweises neutrophiler Granulozyten überlegen war. **Klinische Relevanz:** Die Eosin-Thiazin-Färbung ist mit einer deutlichen Zeitersparnis verbunden und verbessert somit die Praxistauglichkeit der exfoliativen Endometriumszytologie.

Summary

Introduction: Exfoliative endometrial cytology is an easy and valuable diagnostic tool for the detection of inflammatory processes of the uterus that correlates well to culture results. The practical use of this procedure is limited due to the time-consuming, Papanicolaou-Shorr staining technique. In this study the suitability of the rapid Eosin-Thiazin staining for endometrial smears was investigated. **Material and Methods:** Sample collection was carried out with a guarded culture swab (Knudsen-Catheter) in 27 broodmares during routine gynaecological examination. Two smears were prepared from each collection, one was stained according to Papanicolaou-Shorr and the second by the Eosin-Thiazin method (Hemacolor®, Merck). Compared were the presentability of polymorphonuclear neutrophils (PMN) and the calculated PMN scores. **Results:** PMN were easily identifiable in both specimens. In Papanicolaou-Shorr stained smears ten samples showed neutrophil granulocytes (37 %), whereas in the Eosin-Thiazin staining twelve samples were positive (44 %). Thus results corresponded in 25 mares (95 %). **Conclusions:** Eosin-Thiazin staining is a suitable staining method for endometrial smears of broodmares, which surpassed Papanicolaou-Shorr method in two cases. **Clinical relevance:** The use of Eosin-Thiazin staining provides a considerable gain of time and renders endometrial cytology more attractive for routine stud farm practice.

Introduction

Collection of endometrial swabs is one of the most common procedures within the breeding soundness examination of mares. In routine practice the swab is usually prepared for bacteriological examination only (27, 28), even though a good correlation of bacteriological and cytological results exist (5, 13, 15). In some recent investigations sensitivity of cytological results was even superior over bacteriological results (17, 20). The presence of polymorphonuclear neutrophil granulocytes (PMNs) in endometrial smears is a good indicator of endometritis. Inflammation is rapidly detected and results allow an immediate decision whether breeding in the current cycle can be successful. False positive and false negative culture results can challenge the diagnosis of endometritis. Especially for cases of subclinical endometritis the routine diagnostic techniques as ultrasonography and uterine culture offer a low sensitivity (16, 19). Contamination of cervical or vaginal origin or during sample processing can lead to false positive culture results that do not reflect the endometrial status (1, 3, 12). On the other hand, a positive culture result not necessarily correlates with endometrial inflammation. Response to bacterial inoculation of the uterus varies between

individuals, depending on conditions like uterine clearance. Even in mares with a healthy endometrium, a positive culture result is possible (10, 12, 29). In addition to aerobic bacterial infections, anaerobic bacteria, pneumovagina, urine pooling as well as spermatozoa and irritating substances can cause inflammatory endometrial reactions (20). Interpretation of endometrial smears can support the confirmation of diagnosis, especially in mares with chronic nonsuppurative endometritis, which show no bacterial growth. Exfoliative endometrial cytology as diagnostic tool is easy to implement in the gynaecological examination of broodmares. Sample collection for cytology can be added to the collection for bacteriological examination. Due to the time-consuming staining method according to Papanicolaou-Shorr, with a duration of 60 minutes, the endometrial cytology is not a common diagnostic procedure (25). Aim of this study was to test the ability of Eosin-Thiazin staining, which takes three minutes only, for endometrial cytologies.

Material and Methods

Probands of the study were 27 broodmares, presented for breeding soundness examination at the Clinic for Obstetrics, Gynaecology and Andrology of Large and Small Animals, Giessen, Germany. To avoid contamination from the caudal reproductive tract, all endometrial smears were taken with the guarded Knudsen-Catheter (Figure 1). This metallic instrument includes an outer sheath to guard an inner coil, which is pushed forward in the uterus. External genitalia were cleaned and treated with an aseptic solution (Cutasept®, Bode Chemie GmbH, Hamburg) before sample collection. For bacterial culture sampling a cotton swab can be pulled through a hole at the tip of the inner coil. Mucus from the coil is collected for the cytological examination (Figure 2). The closed catheter was guided manually through the cervix in the uterus. The inner coil was then pushed forward and kept in contact with the endometrium for 30 seconds. The catheter was closed before removal from the uterus and two slides were prepared from each catheter. One slide was stained immediately according to Papanicolaou-Shorr and the second was airdried and stained by the Eosin-Thiazin method (Hemacolor®, Merck, Darmstadt) (11). For the Eosin-Thiazin staining (total duration 3 min) slides were dipped 5 times in the three solutions, respectively. Papanicolaou-Shorr staining was more time consuming (total duration 60 min). Slides need to be 15 min in each alcohol concentration, 6 minutes in the Hematoxylin-Solution, and 2 minutes in the Shorr-Solution. The slides were then carefully washed under water, airdried, and covered with a glass slip. Evaluation of the slides was conducted using a light-microscope at 400-fold magnification. For each slide, seven fields of vision were evaluated for the presence of cells and endometrial

cells and neutrophil granulocytes were counted. Based on the total counts of endometrial cells and neutrophil granulocytes, smears were classified according to a modified score of Couto and Hughes (1985) (6), which is summarized in Table 1. Specimens with more than 0.5 % neutrophil granulocytes were classified as positive for endometritis. Results of both staining techniques were compared to each other with regards to the presentation of the cells and the calculated score in either staining.

Results

Both staining techniques resulted in staining permissible for the detection of neutrophil granulocytes and endometrial cells. The appearance of both cell types differed between the tested techniques. Papanicolaou-Shorr staining resulted in endometrial cells with a blue to blue-green cytoplasm and red nuclei. PMNs showed slightly stained pale green cytoplasm with red-brown nuclei (Figure 3). Variations in colour intensity between slides were observed in Papanicolaou-Shorr staining. Endometrial cells following Eosin-Thiazin staining were characterized by a pale purple cytoplasm and a dark purple nucleus (Figure 4). Cytoplasm of neutrophil granulocytes was barely stained, while the nucleus appeared very dark purple (Figure 5). Nonetheless all cells could easily be identified in both staining. Interpretation of the smears resulted in slight differences. Ten broodmares showed PMNs in Papanicolaou-Shorr stained uterine smears (37 %), whereas in the Eosin-Thiazin stained smears 12 samples were PMN positive (44 %). Hence, in two cases > 0.5 % PMNs were detectable in the Eosin-Thiazin staining whereas the corresponding Papanicolou-Shorr stained slide revealed a negative result. In 25 mares results corresponded between the both staining methods (95 %). Three mares presented higher PMN scores in Papanicolaou-Shorr stained smears than in the also positive Eosin-Thiazin stained smears (Figure 6).

Discussion

Papanicolaou staining in the context of exfoliative cytology of the reproductive tract was first introduced by Papanicolaou in 1917 while studying the guinea pig's oestrous cycle (23). This staining procedure for cytological samples was based on wet fixation, followed by hematoxylin and numerous special counterstains (26). The advantage of this technique was the ability to detect the degree of maturity of squamous epithelial cells. With this, the Papanicolaou era in diagnostic cytology was started (4). Nowadays this staining procedure is primarily used for cycle determination by vaginal cytology in various species (7, 24) due to its ability to distinguish between acidophilic and keratinized superficial cells (7). In endometritis

diagnostic it has been referred to as standard staining procedure (25), whereas only seasonal cyclical changes and no classification of cycle stage in horses were detectable with this staining (8, 25). Papanicolaou-Shorr staining is carried out following wet fixation, so that cells show a high transparency and several layers of cells can be evaluated. Acidophile cells appear red-orange, while basophil cells stain blue. In this investigation all smears prepared with Papanicolaou-Shorr staining showed sufficiently coloured cells that were easily identifiable as endometrial cells or PMNs. Variation on colour intensity did not affect this ability. Benefits of the wet fixed stains compared to air dried preparations are a better defined nuclear chromatin and a sharper nuclear outline (14). This disadvantage can be prevented with quick air-drying, which has been reported to avoid artefacts (14). Hand-held fans can help to shorten the air-drying time (2). The Papanicolaou-Shorr staining procedure is able to identify cytoplasmatic keratinisation through the orange G dye, while this is not the case for the Eosin-Thiazin staining. This is an insignificant disadvantage, as the staining in this case is not used for the purpose of oestrus detection. A more important benefit of Papanicolaou-Shorr staining, compared with Eosin-Thiazin staining, is the ability to stain thick three dimensional cell clusters and show well defined, individual, intact, non-necrotic cells (14). This fact can explain the higher degree of PMNs in three mares; in these cases PMNs seemed to be easier to visualize in the Papanicolaou-Shorr staining. On the other hand, in two mares Eosin-Thiazin staining revealed to be superior over the Papanicolaou-Shorr staining in the current study. This could be due to observed variation in staining intensity, which can occur easily in Papanicolaou-Shorr staining, as this complex staining procedure needs intensive care to keep all reagents in optimal condition. Another staining-independent explanation for this discrepancy in two slides could be a difference in PMN presence on the two slides. The manageable disadvantages of the Eosin-Thiazin staining procedure, compared to the Papanicolaou-Shorr staining, stand behind numerous advantages of this technique. The major advantage and reason for preparation of Eosin-Thiazin procedure is the apparent saving of time with this rapid staining procedure. In addition also qualitative improvement of stained smears can be obtained with Eosin-Thiazin staining. Air-drying process leads to an apparent enlargement of cells and nuclei which makes Eosin-Thiazin stained smears preferable for morphometry (21). Recent studies introduced computerized morphometric analysis of epithelial nuclei as an upcoming diagnostic tool to evaluate endometrial cytologies in subfertile bitches (9). It could be worth the effort to test this procedure for automated interpretation of endometrial cytologies in mares. Furthermore cytoplasmatic details are enhanced in Eosin-Thiazin staining and cellular granules are easily identifiable by the Eosin-

Thiazin staining (14). As granulocytes, others than neutrophils, are not a common cell type in endometrial smears of horses not suffering from endometritis, we unfortunately did not have the opportunity to identify this cell type in our investigation. Eosinophils are of particular interest as they commonly can be detected in mares with pneumovagina (22). The granules of eosinophils and mast cells are poorly stained by Papanicolaou-Shorr, while Eosin-Thiazin demonstrates excellent granule details (14). Lastly, mucus is visible in the Eosin-Thiazin staining and can provide information on the secretory status of the endometrium. Results of the current study identify the Eosin-Thiazin staining as appropriate technique for preparation of equine endometrial cytologies. The most important benefit is the fast availability of results. This gives the opportunity to detect uterine inflammation immediately and helps to decide whether to breed or not to breed the mare in the current oestrous cycle in the case of suspect endometritis (18). Main indicator for equine endometritis are PMNs, which are easily identifiable with this staining. For multiple layer smears, Papanicolaou-Shorr staining still remains superior.

Conclusions

Results of the study prove the suitability of Eosin-Thiazin staining for equine endometrial cytologies. The benefit of time, compared with the Papanicolaou-Shorr staining, helps to accommodate this diagnostic procedure in the routine stud farm medicine.

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Table 1: Classification of cytologcial results with a modified score according to Couto and Hughes (1985) (6).

Score	Percentage of neutrophile granulocytes	
-	0 - 3 %	neutrophile granulocytes
+	3 - 10 %	neutrophile granulocytes
++	> 10 - 30 %	neutrophile granulocytes
+++	> 30 - 50 %	neutrophile granulocytes
++++	> 50 - 75 %	neutrophile granulocytes
+++++	> 75 %	neutrophile granulocytes